

Attorney Docket No.: PTQ-0027
Inventors: Van Eyk et al.
Serial No.: 09/115,589
Filing Date: July 15, 1998
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Remarks/Arguments begin on page 3.

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REMARKS

Claims 80-84 and 87-98 are pending in the instant application.

In the Telephone Interview conducted on August 28, 2006, Applicants were advised that the amendment filed August 18, 2006 was responsive to the Restriction Requirement and overcame the objections to the specification under 35 U.S.C. 132.

Further, with respect to the rejection of claims 80, 81, 87, 88, 89, 90, 91, 92, 93, 94, 95 and 96 under 35 U.S.C. 102(a) as being anticipated by Takahashi et al. and the rejection of claims 80-84 and 87-98 under 35 U.S.C. 103(a) as being unpatentable over Takahashi et al., as applied to claims 80, 81, 87, 88, 90, 91, 92, 93, 94, 95 and 96 in Section III, supra and further in view of Westfall et al., the Examiner acknowledged that Takahashi et al. do not teach or suggest detecting TnI peptide fragments for assessing skeletal muscle damage. However, the Examiner suggested that, absent evidence otherwise, the assay of Takahashi et al. may inherently detect TnI peptide fragments and thus anticipate and/or render obvious the claimed invention.

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Accordingly, in an earnest effort to advance the prosecution of this case, Applicants are submitting evidence herewith, per the Examiner's request, demonstrating that the assay of Takahashi et al. does not detect peptide fragments of a skeletal myofilament protein as claimed, and in particular a peptide fragment of skeletal TnI.

At page 302 of Takahashi et al. they teach an optimal monoclonal antibody combination for ELISA of MAb F1-17 as the capture antibody and MAb 3I-35 as the detector antibody for detecting skeletal TnI.

Applicants are submitting herewith a reference by Simpson et al. published in 2000 in Journal of Applied Physiology which demonstrates that MAb 3I-35 does not detect a known peptide fragment of skeletal TnI, even with prolonged exposure of film to chemiluminescent western blot. Applicants respectfully direct the Examiner to Figure 2 at page 756 and teachings in col.1 of page 756 of Simpson et al. (J. App. Physiol. 2000 88:753-760) showing the failure of MAb 3I-35 to reveal the 17.5 kDa peptide fragment of skeletal TnI indicative of skeletal muscle damage. The Simpson et al. paper thus shows that the assay taught by Takahashi et al. is not enabling for detecting a skeletal TnI peptide fragment as required in order to be an anticipatory reference under 35 U.S.C. 102. Further, the

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Simpson et al. paper establishes that the "missing descriptive matter" in Takahashi [namely any teaching or suggestion of detecting a peptide fragment for assessing skeletal muscle damage] is NOT "necessarily present" in the assay of Takahashi and thus is NOT inherent in the assay of Takahashi et al. (see MPEP 2112).

Accordingly, since the Examiner has acknowledged that there is no teaching or suggestion in the cited references of detecting TnI peptide fragments for assessing skeletal muscle damage and since the evidence submitted herewith, per the Examiner's request, demonstrates that detecting a peptide fragment of skeletal TnI is neither enabled nor inherent in teachings of Takahashi et al., withdrawal of the rejections under 35 U.S.C. 102(a) and 35 U.S.C. 103(a) is respectfully requested.

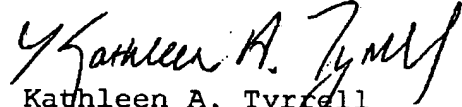
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Conclusion

Applicants believe that the foregoing comprises a full and complete response to the Telephone Interview of August 28, 2006. Accordingly, favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,


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Hypoxemia-induced modification of troponin I and T in canine diaphragm

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Simpson, Jeremy A., Jennifer E. Van Eyk, and Steve Iscoe. Hypoxemia-induced modification of troponin I and T in canine diaphragm. *J. Appl. Physiol.* 88: 753–760, 2000.—Impaired muscle function (fatigue) may result, in part, from modification of contractile proteins due to inadequate O₂ delivery. We hypothesized that severe hypoxemia would modify skeletal troponin I (TnI) and T (TnT), two regulatory contractile proteins, in respiratory muscles. Severe isocapnic hypoxemia (arterial partial pressure of O₂ of ~25 Torr) in six pentobarbital sodium-anesthetized spontaneously breathing dogs increased respiratory frequency and electromyographic activity of the diaphragm and internal and external obliques, with death occurring after 131–285 min. Western blot analysis revealed proteolysis of TnI and TnT, 17.5- and 28-kDa fragments, respectively, and higher molecular mass covalent complexes, one of which (42 kDa) contained TnI (or some fragment of it) and probably TnT in the costal and crural diaphragms but not the intercostal or abdominal muscles. These modifications of myofibrillar proteins may provide a molecular basis for contractile dysfunction, including respiratory failure, under conditions of limited O₂ delivery.

respiratory muscles; contractile proteins; protein degradation; respiratory failure; protein modification

RESPIRATORY FATIGUE IS ASSOCIATED with the decreased ability of respiratory muscles to generate the pressures required to maintain ventilation. Rather than maintaining ventilation, individuals will tolerate the fall in arterial partial pressure of O₂ (P_{aO₂}) and rise in arterial partial pressure of CO₂ (P_{aCO₂}), which accompany hypoventilation. In individuals with acute exacerbations of obstructive diseases, hyperinflation also ensues; the resulting decrease in diaphragmatic length and increased radius of curvature place it at a mechanical disadvantage. Any decrease in contractile efficiency combined with decreased energy supply (reduced P_{aO₂}) only makes the diaphragm (and other respiratory muscles) more susceptible to eventual failure (for reviews, see Refs. 24 and 32).

Diagnosis of respiratory muscle dysfunction typically involves measurements of either electrical activity of the diaphragm or pressures generated during maximal voluntary efforts, sniffs, or magnetic stimulation of the

phrenic nerves (see Refs. 11, 16, 24, 32). Although some of these methods have distinct advantages (e.g., the simplicity of the sniff test), others are either technically demanding (e.g., magnetic stimulation, computation of power spectrum of the electromyogram) and/or are invasive (placement of catheters in the esophagus or abdomen). Moreover, they all share one major limitation: insensitivity; all require that enough damage be present to result in a significant reduction in, for example, pressure. Plasma levels of creatine kinase (CK), a marker of cellular necrosis, have yet to be established as a useful marker of respiratory failure (1, 10).

Although plasma levels of the fast and, possibly, slow skeletal isoforms of troponin I (sTnI) increase following severe exercise (22, 27, 28), it is unclear if intact sTnI or some unidentified modification product(s) was measured. Indeed, in one study (28), only the fast isoform was measured. Hence, we do not know the extent of cell damage, including necrosis, responsible for leakage of the protein, intact or modified, into the plasma. In contrast, plasma levels of two myocardial proteins, cardiac troponin I and troponin T (cTnI and cTnT), can now be rapidly measured, with their levels indicating the extent of infarction (see Ref. 7). Myocardial ischemia-reperfusion injury in isolated rat hearts has recently been shown to be associated with modification of cTnI (12, 20, 31); in fact, the extent and nature of cTnI modification are related directly to the severity of the ischemia-reperfusion injury and the fall in force generation (12, 31). Even under conditions that involve little or no loss of protein, indicating minimal cellular necrosis, cTnI undergoes degradation and covalent complex formation (20). Thus cTnI modification within the myocyte may therefore be the earliest marker of myocardial damage. With more severe injury, other myofilament proteins, including cTnT, are also modified (e.g., Refs. 14, 20, 31). Thus modification products of myofilament proteins may provide useful indexes of the severity of injury.

Plasma levels of sTnI and sTnT cannot, however, be used to diagnose respiratory muscle dysfunction until confirmation of both their modification within tissues and their release into the plasma. In this study, we addressed the first issue by testing the hypothesis that severe hypoxemia would modify myofibrillar proteins of respiratory muscles of anesthetized, spontaneously breathing dogs. Our results show that both TnI and TnT were modified but only in diaphragm; these modifications consisted of lower molecular mass degradation

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products and higher molecular mass covalent complexes consisting of TnI, TnT, and possibly troponin C (TnC).

METHODS

Experiments were conducted according to procedures established by the Canadian Council on Animal Care and after approval by the Animal Care Committee of Queen's University. Six mongrel dogs (2 males, 4 females; weight of 16–24.4 kg, mean 19.8 kg) were anesthetized with an intravenous injection of pentobarbital sodium (35 mg/kg), which was supplemented if the animals displayed a brisk response to noxious stimulation of a toe pad or blinked in response to stimulation of the cornea. In brief, after instrumentation and collection of control tissue samples (see below), they breathed a hypoxic gas mixture; measurements of cardiorespiratory parameters were made at 20-min intervals until signs of impending death appeared. They were then mechanically ventilated, and biopsies were taken from a limb muscle and various respiratory muscles for subsequent analysis of troponin modification.

Surgical preparation included insertion of an endotracheal tube to which a heat and moisture exchanger was attached, a venous "butterfly" cannula into a forelimb vein for administration of supplemental anesthetic, and a cannula into the carotid artery for measurement of arterial blood pressure and withdrawal of blood for measurements of arterial blood gases. Two Swan-Ganz catheters (131HF7, Baxter Edwards, Deerfield, IL) were inserted via the right jugular vein, one into the pulmonary artery and the other into the right atrium, according to measurements of pressure at the tip of each catheter. The former was used for sampling mixed venous blood, and both were used for thermal dilution measurements of cardiac output (Qr) when cold saline was injected into the latter (Edwards 9520 cardiac output computer, Santa Ana, CA).

Tissue Samples

Before imposition of hypoxemia, control biopsies were taken from the quadriceps and internal oblique (IO) muscles. To gain access to the IO, the aponeurosis of the overlying external oblique (EO) was incised and the muscle was reflected laterally. Samples at the end of the experiment were taken from quadriceps, EO, and IO as well as the transverse abdominis, costal and crural diaphragms, and caudal and midthoracic (intercostal spaces 9–10 and 4–6, respectively) external and internal intercostals. Tissue samples were obtained before the heart stopped or within 4 min of death (cessation of the heart beat). They were immediately washed in cold saline, frozen in liquid N₂, and stored at –70°C until later biochemical analysis (see *Biochemistry*, below).

Electromyographic Recordings

After the first biopsies were taken, pairs of fine wires that were insulated except for the tips, which were bent back over the outside of 23-gauge needles, were inserted under direct observation ~15 mm apart into the IO on the side opposite to that from which the biopsy had been made. The needles were then withdrawn, leaving the wires in place. Similar electrodes were placed into the ipsilateral EO. The overlying skin was then closed. Recordings of the diaphragmatic electromyograph (EMG) were made by inserting identical electrodes percutaneously at the sixth or seventh right interspace. All signals were amplified and filtered (Grass P511J, Quincy, MA); signals were recorded on videotape after pulse code modulation (NeuroCorder DR886, New York, NY) and re-

corded on paper (Gould TA2000, Cleveland, OH), either as raw signals or after "integration" (Paynter filter, time constant of 100 ms).

Hypoxemia

Severe "isocapnic" hypoxemia was instituted by having the dogs breathe through a circuit that maintains normocapnia regardless of the level of hyperpnea (26). In brief, dogs inhaled a gas mixture of 9.5% O₂-balance N₂ delivered at ~2 l/min to a balloon attached to a one-way valve on the inspiratory side of the breathing circuit. The remaining gas inspired during hypoxia-induced hyperpnea came from a cylinder containing 9.5% O₂-6.5% CO₂-balance N₂ and connected to a demand valve (Dacor, Northfield, IL). Thus any hypoxia-induced demand for ventilation greater than the basal flow was supplied by gas from the second cylinder, which, because of its CO₂ content, prevented hypocapnia.

Protocol

Two sets of control measurements, 20 min apart, were taken of arterial and mixed venous blood gases and pH (Radiometer ABL30, Copenhagen, Denmark) and Qr. The dogs were then placed on the breathing circuit. Once the PaO₂ had fallen to the desired range (24–28 Torr), measurements were repeated at 20-min intervals until the dogs died. Death was always preceded by a sudden slowing of respiratory frequency, at which time dogs were ventilated with room air and biopsies were taken as described.

Biochemistry

Plasma levels of CK before and at the end of hypoxia exposure were determined (Beckman Synchron CX, Fullerton, CA). For SDS-PAGE analysis, frozen tissue samples were homogenized in 50 mM Tris, pH 7.8, plus a cocktail of protease inhibitors (50 μM phenylmethylsulfonyl fluoride, 3.6 μM leupeptin, 2.1 μM pepstatin A, and 10 mM EDTA). Total protein concentration was determined by the Lowry assay before preparation of protein samples in Laemmli buffer and 1 mM dithiothreitol (DTT) and storage at –20°C (20). SDS-PAGE (12.5%) and Western immunoblots were performed as previously described (20) using mini-Protein II and wet transfer mini-systems (Bio-Rad, Hercules, CA). Gels were stained with Coomassie blue or transferred to nitrocellulose (27 V for 16 h). Nitrocellulose blots were transiently stained with ponceau S to identify molecular mass markers and then incubated in blocking solution [50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (vol/vol) Nonident P-40, 0.25% (wt/vol) gelatin, and 3% (wt/vol) BSA]. Detection of slow and fast sTnI was done using anti-TnI monoclonal antibody (MAb) clones C5 (Research Diagnostics, Flanders, NJ), 8I-7 and 3I-35 (Spectral Diagnostics, Toronto, ON), and 10F2 (Sanofi, Montpellier, France). (All MABs cross-react, but to different degrees, with the three isoforms of TnI; the cardiac isoform of TnI is absent in skeletal muscle.) The various anti-TnI MABs were epitope mapped according to Van Eyk et al. (31). Detection of TnT was done using three anti-TnT MAB clones: JLT-12 (Sigma, Mississauga, ON), 4D11 (Biodesign, Kennebunk, ME), and 4TnT conjugated to horseradish peroxidase (Spectral Diagnostics, Toronto, ON). All displayed cross-reactivity to the skeletal and cardiac isoforms. The only MAB to cross-react with another muscle protein was the anti-TnI MAB 3I-35 (~10% with sTnT).

Blots were incubated with anti-mouse IgG antibody-alkaline phosphatase conjugate (Jackson Laboratories, West Grove, PA) and detected by CDP-Star chemiluminescence (NEN-Mandel, Boston, MA) except for 4TnT conjugated to

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Table 1. *Cardiorespiratory parameters before and midway and for last set of complete measurements before death during hypoxemia*

	Time 0	Time 0.5	Time 1
f, min ⁻¹	9.0 ± 4.2	49.3 ± 17.1*	44.5 ± 10.4*
peak fDia, AU	6.1 ± 2.5	14.1 ± 8.5*	10.5 ± 6.1*
peak fEO, AU	1.6 ± 1.2	5.8 ± 2.2	5.4 ± 3.4
peak fIO, AU	1.7 ± 1.8	10.2 ± 2.0*	10.4 ± 3.2*
QT, l·min ⁻¹ ·kg ⁻¹	132 ± 24	161 ± 46	200 ± 82
PaO ₂ , Torr	64.9 ± 4.5	24.7 ± 2.8*	19.8 ± 3.3*†
Paco ₂ , Torr	34.2 ± 6.7	35.3 ± 6.7	42.1 ± 9.6
pHa	7.310 ± 0.060	7.280 ± 0.070	7.241 ± 0.094
PvO ₂ , Torr	43.9 ± 6.5	15.4 ± 1.3*	10.9 ± 2.4*
PvCO ₂ , Torr	36.8 ± 8.9	43.4 ± 9.2	49.7 ± 8.6
pHv	7.282 ± 0.027	7.270 ± 0.074	7.220 ± 0.100
CaO ₂ , ml O ₂ /dl	15.8 ± 0.10	6.8 ± 1.2*	4.8 ± 2.3*†
CvO ₂ , ml O ₂ /dl	11.8 ± 1.9	3.6 ± 0.9*	1.9 ± 1.2*
DO ₂ , ml O ₂ ·kg ⁻¹ ·min ⁻¹	20.9 ± 4.3	10.8 ± 3.1*	8.5 ± 2.7*
VO ₂ , ml O ₂ ·kg ⁻¹ ·min ⁻¹	5.15 ± 1.98	5.09 ± 1.98	5.95 ± 1.11
Hct	37.9 ± 1.9	43.6 ± 5.3*	45.7 ± 5.6*
Creatine kinase, U/l	170 ± 32		360 ± 163*

Values are means ± SD. Time 0, time 0.5, and time 1, measurements before, midway, and immediately before death; f, respiratory frequency; peak fDia, peak integrated diaphragmatic activity; peak fEO, peak integrated external oblique activity; peak fIO, peak integrated internal oblique activity; AU, arbitrary units; QT, cardiac output; DO₂, oxygen delivery; PaO₂, arterial partial pressure of O₂; Paco₂, arterial partial pressure of CO₂; PvO₂ and PvCO₂, mixed venous PO₂ and PCO₂; CaO₂ and CvO₂, arterial and venous O₂ content; Hct, hematocrit. *Significantly different from control, $P < 0.05$; †significantly different from time 0.5, $P < 0.05$. One-way repeated-measures ANOVA with Bonferroni post hoc analysis or, for nonparametric data, Friedman's repeated measures ANOVA with Student-Newman-Keuls post hoc analysis was performed.

horseradish peroxidase, which was detected directly without use of a secondary antibody. The quantities of intact TnI or TnT and the various modification products were determined by densitometry (33). Standard curves of purified fast TnI were run to ensure that densitometric measurements for both Coomassie blue stains and Western blots using C5 MAb were within the linear range of detection. The quantities of TnI or TnT modification products were normalized by expressing their densitometric values as a fraction of the combined values of the slow and fast isoforms of intact TnI. Results were analyzed by paired *t*-tests or one-way repeated mea-

sures ANOVA, with post hoc analyses, as appropriate, to determine if differences were significant ($P < 0.05$).

RESULTS

Inhalation of the hypoxic gas mixture resulted in significant decreases in mean PaO₂ (from ~65 to 25 Torr), arterial O₂ content, and mixed venous PO₂ (Table 1). Increases in QT did not reach statistical significance nor did they, in combination with a significant increase in hematocrit, prevent a drop in whole body O₂ delivery (DO₂). Peak amplitudes of integrated EMG activity of the diaphragm and IO as well as respiratory frequency increased. Death occurred after an average 197 min (range 131–285 min) of breathing the hypoxic gas mixture; in all animals, death was preceded by a cessation of EMG activity in all respiratory muscles. In four dogs, all muscles stopped firing at the same time, but, in the other two, EO stopped firing first by ~2 min. Plasma CK levels, a marker of cell necrosis, increased significantly, but its source(s) was not identified.

Myofibrillar Proteins in Respiratory Muscles

TnI. All respiratory muscles contained bands (Fig. 1) corresponding to the fast and slow isoforms of sTnI (~25 and ~26 kDa, respectively, on 12.5% SDS-PAGE, compared with their actual molecular masses of 21.2 and 21.6 kDa), which were identified using samples from exclusively fast (caudofemorals) and slow (soleus) muscles of cat (Fig. 1A). (The faint band representing a degradation product in soleus is due to ischemia of this particular muscle.) However, only the costal and crural diaphragms showed evidence of hypoxemia-induced degradation of TnI, evident as a band at a position corresponding to a molecular mass of 17.5 kDa. Samples of nonhypoxemic diaphragms from two dogs used in other experiments showed no evidence of TnI degradation (Table 2); other nondiaphragmatic respiratory muscles (e.g., IO; Fig. 1B) also showed no evidence of this. The hypoxemic crural diaphragm of dog 6 also yielded the 17.5-kDa degradation product despite the virtual absence of the band corresponding to the fast

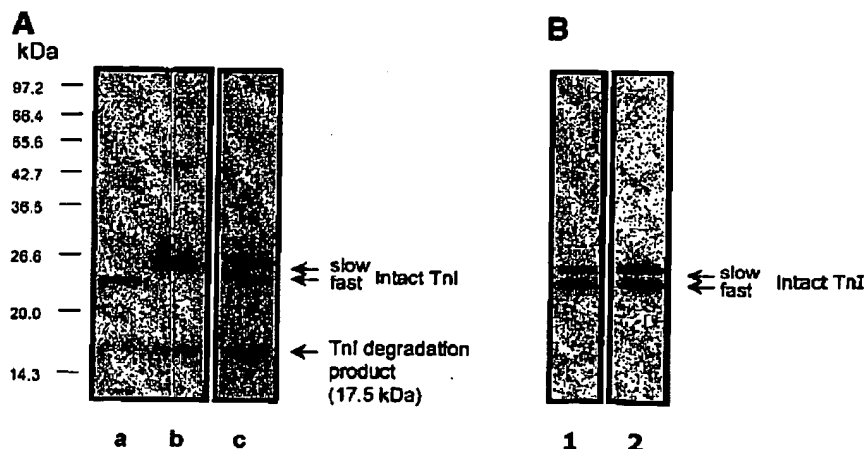


Fig. 1. A: Western blots using anti-troponin I (TnI) monoclonal antibody (MAb) C5 of biopsies from a costal diaphragm (lane c) of a severely hypoxemic dog. Feline caudofemorals (lane a) and soleus (lane b) were used to identify the fast and slow isoforms, respectively, of skeletal TnI. B: Western blots using anti-TnI MAb C5 of internal obliques (IO) before (lane 1) and after (lane 2) severe hypoxemia. Degradation of TnI was <2% in all samples of IO.



Table 2. Hypoxemia-induced degradation of TnI

	Control	Hypoxemia
Diaphragm (costal)	<2*	20.0 ± 8.9
Diaphragm (crural)		26.1 ± 8.4
Internal oblique	<2	<2
External oblique		5.8 ± 3.6
Transverse abdominis		2.9 ± 1.9
Internal intercostal 4		2.1 ± 1.3
External intercostal 4		3.4 ± 1.6
Intercostal 10		2.4 ± 0.8
Quadriceps	2.6 ± 4.2	2.0 ± 1.7

Values are means ± SD. Percent degradation expressed as densitometric "volume" of 17-kDa fragment divided by the sum of the volumes of the fast and slow isoforms of intact troponin I (TnI). *Based on samples from 2 dogs not used in these experiments.

isoform of intact sTnI (Fig. 1A), suggesting that the degradation product originated from, but not necessarily exclusively, slow TnI. This same dog, however, had almost equal amounts of the fast and slow isoforms of TnT (data not shown).

Western blots using anti-TnI MABs C5 and 3I-35 of representative hypoxemic diaphragms and an accessory respiratory muscle, the IO, are shown in Fig. 2. They consistently and unambiguously revealed, particularly with prolonged exposures, a higher molecular mass covalent complex only in the diaphragm (compare lanes c and d in Fig. 2). The failure of the MAB 3I-35, which binds to the extreme COOH terminus (20), to reveal the 17.5-kDa fragment, even with prolonged exposure, indicates that formation of the 17.5-kDa fragment involves proteolysis at the COOH terminus. In contrast, the binding of this MAB to the 42-kDa covalent complex indicates that this complex contains TnI with an intact COOH terminus. The higher molecular mass complexes did not dissociate in the presence of 6 M urea or 1 mM DTT (data not shown), indicating that they are covalent (nondisulfide) in nature.

We believe that the two bands beneath the 42-kDa complex, most visible in lane e (Fig. 2), result from cross-reactivity of 3I-35 to the intact slow and fast

isoforms of TnT for three reasons: 1) only this anti-TnI antibody detected the bands, 2) this MAB also detected TnT in purified troponin, albeit with reduced affinity (~10%, data not shown), and 3) the two bands aligned perfectly with the slow and fast isoforms of intact TnT revealed with the anti-TnT MABs JLT-12 (Fig. 3) or 4D-11.

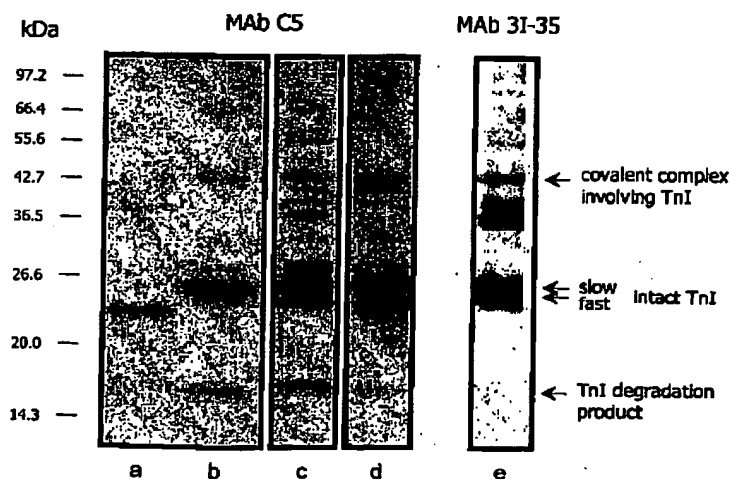
The extent of TnI degradation in biopsies from all respiratory muscles, expressed as a percentage of the sum of the intact fast and slow TnI isoforms, is presented in Table 2. Hypoxemia-induced degradation was present in the diaphragms of all dogs, averaging 20% and 26% in the costal and crural diaphragms, respectively, but did not exceed 6% in any other respiratory muscle or the quadriceps. There was no significant difference ($P > 0.05$, paired *t*-test) between the amount of TnI degradation product in the costal and crural diaphragms (in the five dogs in which samples were available from both parts), suggesting no regional differences in susceptibility to damage. Moreover, because the crural biopsy was always done after the costal one, its later removal did not result in more degradation.

TnT: Different patterns of TnT modification products were detected using three different anti-TnT MABs (Fig. 3). 4TnT (data not shown) and JLT-12 detected only a single TnT degradation product with a molecular mass of 28 kDa. Although JLT-12 revealed the degradation fragment in three of five crural and in two of six costal diaphragms, another MAB, 4D-11, detected formation of two high molecular mass (42 and 66 kDa) complexes in all hypoxemic diaphragms, costal and crural, but not in any other respiratory muscle. However, confirmation that TnT is part of these complexes requires its detection by at least one other MAB with a different epitope.

DISCUSSION

This study is the first, to our knowledge, to report hypoxemia-induced posttranslational modification of

Fig. 2. Western blots using anti-TnI MABs C5 and 3I-35 of representative hypoxemic diaphragms (lanes c and d) and an accessory respiratory muscle (IO; lane e). Feline caudofemoralis (lane a) and soleus (lane b) were used to identify the fast and slow isoforms, respectively, of skeletal TnI. Prolonged exposure revealed a higher molecular mass covalent complex in the diaphragm (lane c) but not the other respiratory muscles represented by IO (lane d). MAB 3I-35, which binds to the extreme COOH terminus, did not reveal the 17.5-kDa fragment even with prolonged exposure (lane e) but did reveal the 42-kDa covalent complex, indicating that the complex contains TnI with an intact COOH terminus.



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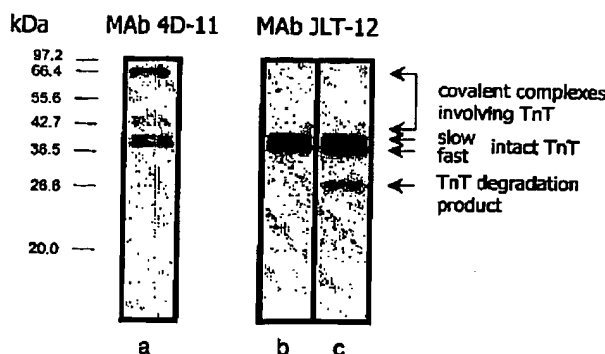


Fig. 3. Western blots using anti-troponin T (TnT) MAb 4D-11 and JLT-12 of a representative crural diaphragm (lanes a and c) and IO (lane b) of severely hypoxemic dog. MAb 4D-11 revealed the presence of higher molecular mass complexes only in diaphragms, whereas JLT-12 revealed only a lower molecular mass degradation product and only in diaphragm.

identified myofibrillar proteins (TnI and TnT) in a skeletal muscle. The acute changes observed in our dogs would precede any transcriptional changes such as isoform switches. These posttranslational modifications may, however, account for the impaired performance of respiratory muscles in a variety of acute, and possibly chronic, clinical conditions (for reviews, see Refs. 24 and 32).

Hypoxemia was induced by lowering $F_{I_{O_2}}$. Cessation of respiratory activity and death occurred after an interval similar to that in dogs in which diaphragmatic fatigue was induced by reducing whole body DO_2 with cardiac tamponade (2). However, without measurements of regional perfusion, we cannot be certain that diaphragmatic DO_2 delivery was, indeed, reduced. Although whole body DO_2 averaged 10.8 and 8.5 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$ at the midpoint and end, respectively, of hypoxemic stress, values similar to the critical value (~ 10 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$) for DO_2 during hypoxia (6) but only a fifth of that (~ 50 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$) for the contracting canine diaphragm (34), cessation of respiratory activity was probably not due to failure (fatigue) of the respiratory muscles for four reasons. 1) It was preceded by a sudden slowing of respiratory frequency, indicating a change in output of the central respiratory controller. 2) Activity of all muscles ceased simultaneously in four of the six dogs. 3) Protein modification occurred only in the diaphragm; had the diaphragm failed, other muscles would have compensated, possibly leading to hypoxemia-induced protein modification in them. 4) Institution of mechanical ventilation elicited a transient recovery of activity in all muscles. Respiratory arrest and death therefore appear to have been due to central (brain stem) damage resulting from severe prolonged hypoxemia, a conclusion consistent with that of a previous report (35).

TnI and TnT Modifications and Contractile Function

Hypoxemia-induced modifications of TnI and TnT in tissue consisted of both lower molecular mass (17.5 and

28 kDa, respectively) fragments and higher molecular mass complexes (42 and 66 kDa). Modification products other than these and detectable using MAb with different epitopes may, however, also be present. Although misidentification is possible due to cross-reactivity of MAb with other muscle proteins, all products except covalent complexes possibly containing TnT were recognized by MAb with different epitopes to the same protein, a finding that greatly reduces the probability of misidentification. Nevertheless, the observation that TnI and TnT were modified only in the diaphragm indicates both that the MAb we used are specific to acute muscle injury and that, under our experimental conditions (severe hypoxemia), the diaphragm was the sole muscle susceptible to such modification.

Differential binding of the anti-TnI MAb C5, 3I-35, and 10F2 to intact TnI and the 17.5-kDa degradation product suggests initial proteolysis of sTnI at the COOH terminus. We mapped the TnI fragments using different MAb; whereas C5 bonded equally well to all sTnI modification products, 3I-35 and 10F2 bonded only to intact sTnI, indicating that the 17.5-kDa sTnI fragment resulted from proteolysis at or near amino acid residue 162. Although the calculated molecular mass of fragments 1–162 of the slow and fast isoforms are 18.98 and 19.01 kDa, respectively (greater than the measured 17.5 kDa), some proteins, including the troponins, do not migrate according to their molecular masses. The fast and slow isoforms of sTnI migrated "higher" (25 and 26 kDa) than predicted by their actual molecular masses (21.2 and 21.6 kDa, respectively), suggesting that the 1–162 fragment also migrates higher than calculated. Interestingly, Farah et al. (9) observed that the recombinant fast sTnI 1–156 mutant, only six amino acids less, migrated to 22 kDa instead of the expected ~ 19 kDa. These findings indicate that the degradation of sTnI to a 17.5-kDa fragment is too great to be accounted for by anything other than cleavage. The failure of MAb 3I-35 and 10F2 to bind to the fragment could be due to COOH-terminal clipping or block of the epitope at the COOH terminus by posttranslational modification (e.g., phosphorylation, glycosylation). Alternatively, clipping could have occurred first at the NH_2 terminus, with binding of the MAb 3I-35 and 10F2 being blocked by posttranslational modification(s) at the extreme COOH terminus.

Covalent Complexes

Two complexes of 42 and 66 kDa formed in the diaphragm during hypoxemia (Figs. 2 and 3). These involve covalent bonds between the proteins because they were stable in the presence of high concentrations (0.1%) of SDS and in 6 M urea, conditions that disrupt noncovalent interactions, and of the reducing agent DTT (1 mM), which also disrupts disulfide bonds. The 42-kDa product is comprised of TnI and possibly TnT. Importantly, the anti-TnI MAb 8I-7, C5, and 3I-35 identified this complex, indicating that the complex is comprised of TnI with an intact COOH terminus. On the basis of the molecular mass of the complex, further



degradation of TnI (from the NH₂ terminus) or TnT (from either the NH₂ or COOH terminus) probably occurred, reducing the molecular mass of the smaller complex from the calculated 49.5 kDa.

The 66-kDa complex may be composed of fragments of one or more of the three troponin subunits (TnI, TnT, and TnC); the calculated molecular mass of the 17.5-kDa TnI fragment and intact TnT and TnC is ~67 kDa. However, because none of the anti-cTnI MAbs (with different epitopes) bonded to this 66-kDa complex, it either does not contain TnI or the epitopes for the various TnI MAbs antibodies are inaccessible. The 66-kDa complex is also unlikely to be a dimer of TnT due to the physical separation between "adjacent" TnTs on the thin filament. Instead, this protein is likely a covalent complex of TnT and TnC or other unidentified proteins. Interestingly, McDonough et al. (20) have identified cTnC in covalent complexes in ischemic/reperfused rat myocardium.

Skeletal Muscle Proteolysis

To our knowledge, there have been no previous studies of hypoxemia-induced modifications of myofibrillar proteins of respiratory muscles. Indirect evidence, however, suggests that loads modify myofibrillar proteins of respiratory muscles. Reid et al. (23) recovered ~7% less myofibrillar protein (including any TnI and TnT associated with the actin filament) from the diaphragms of hamsters with tracheal banding (compared with ~23% modification of TnI in the diaphragms of our hypoxemic dogs); tropomyosin and α -actinin of loaded diaphragms also appear to have been degraded more quickly by calpain. The latter observation suggests that several myofilament proteins were modified, making them more susceptible to degradation by calpain. Recently, the same group (15) demonstrated increased calpain-like activity in the costal diaphragms of rabbits subjected to a moderate inspiratory resistive load.

There are few descriptions of posttranslational modifications of myofilament proteins in skeletal muscle. Belcastro et al. (4), based on SDS-PAGE of myofibrils from fatigued plantaris of rats, described loss of a 58-kDa band (possibly desmin, a cytoskeletal protein) and the appearance below actin (~42 kDa) of an unidentified protein in the region of troponin and tropomyosin. Belcastro (3) later described faster degradation of tropomyosin and α -actinin from purified myofibrils of hindlimb muscles from exercised rats. Eccentric contraction-induced injury of mouse soleus in vivo increases the rate of proteolysis (affected proteins not identified) by ~60% at 48 h postinjury (19), whereas eccentric contractions of rabbit extensor digitorum longus cause rapid (<15 min) myofilament damage in fast glycolytic fibers, characterized by loss of desmin (18). Together, these results suggest a spectrum of exercise-induced damage to myofilaments and that, with time, there is increasing degradation or loss of specific myofilament proteins.

Plasma levels of sTnI, analyzed using immunoenzymometric assays, along with "traditional" markers like

CK, have recently been used to assess exercise-induced skeletal muscle damage in humans. Rama et al. (22), on the basis of the increases and time course of changes in both sTnI and CK levels after a triathlon, concluded that sTnI is superior to CK as a marker because of its greater sensitivity and faster return to control values. At the end of a marathon, plasma levels of sTnI, measured using the anti-TnI MAb 3I-35, were elevated in only 9 of 46 runners (28); however, since this MAb does not detect the 17.5-kDa TnI degradation product (Fig. 2), the incidence of muscle damage may have been underestimated, especially because, at least in ischemic cardiac muscle, cTnI modification products, not intact cTnI, are preferentially released (20). Although the appearance of sTnI in the blood is a promising marker of muscle injury, these studies demonstrate only that exercise causes membrane damage sufficient to allow the release of muscle proteins. Moreover, because we do not yet know what form of sTnI was measured, the assay may not be quantitative. Until more is known about the changes in sTnI and other skeletal muscle proteins during muscle injury and what forms are released, the utility of sTnI as a marker of damage is limited.

The precise nature of these modifications and their similarity to the changes observed in our canine model of hypoxemia remain to be elucidated. The latter differ, however, from the transformation to the slow isoforms observed in the diaphragms of patients with congestive heart failure (30) and chronic obstructive pulmonary disease (17), both a form of diaphragmatic "exercise" (5). Transformation provides no information about the mechanisms underlying injury and fatigue nor can it serve as a marker of rapid changes in clinical status. This could reflect, in part, differences between acute and chronic injury.

These posttranslational modifications of sTnI and sTnT are similar in some respects to those recently described for cTnI in the ischemic-reperfused isolated rat heart (12, 20). In that model, as the severity of ischemic-reperfusion injury increases from reversible to irreversible, contractile function deteriorates in association with progressive degradation of cTnI (31) and the formation of covalent complexes between its fragments and cTnT or cTnC; little if any intact cTnI is released (20). In the present study, we have shown for the first time that severe hypoxemia initiates similar processes in canine diaphragm. The functional consequences of protein modification in the heart and respiratory muscles, however, likely differ. Protein modification in the heart impairs contractile function and, therefore, myocardial performance because the heart contracts as a syncytium. In contrast, protein modification in some diaphragmatic (or any other respiratory muscle) fibers need not impair ventilation if other motor units within the diaphragm and/or other respiratory muscles compensate. Thus protein modification may occur well before any ventilatory impairment.

The molecular mechanisms and the enzymes responsible for TnT and TnI modifications in the diaphragm are unknown. However, in cardiac muscle during ische-



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mia-reperfusion, calpain, the Ca^{2+} -dependent protease, and Ca^{2+} -dependent tissue-specific transglutaminase are believed to be involved (e.g., Refs. 8 and 20) and activated by the increase in cytoplasmic Ca^{2+} during prolonged myocardial ischemia (25) or on reperfusion (29). Hypoxia-induced changes in cytoplasmic Ca^{2+} in skeletal muscle are unknown, but in some types of smooth muscle (13) and myocardium (21) there is a late influx of Ca^{2+} followed by a large Ca^{2+} burst on reoxygenation, implying the operation of same or similar cellular processes.

In conclusion, severe hypoxemia caused modification of sTnI and sTnT, evident as both 17.5- and 28-kDa fragments, respectively, and 42- and 66-kDa covalent complexes, in the diaphragms of spontaneously breathing anesthetized dogs. Modification was not evident in other respiratory muscles. These modifications may underlie skeletal (including respiratory) muscle dysfunction (fatigue) associated with various acute conditions.

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REFERENCES

1. Abe, K., K. Kobayashi, K. Chida, N. Kimura, and K. Kogure. Dominantly inherited cytoplasmic body myopathy in a Japanese kindred. *Tohoku J. Exp. Med.* 170: 261-272, 1993.
2. Aubier, M., T. Trippenbach, and C. Roussos. Respiratory muscle fatigue during cardiogenic shock. *J. Appl. Physiol.* 51: 499-508, 1981.
3. Belcastro, A. N. Skeletal muscle calcium-activated neutral protease (calpain) with exercise. *J. Appl. Physiol.* 74: 1381-1386, 1993.
4. Belcastro, A. N., W. Parkhouse, G. Dobson, and J. S. Gilchrist. Influence of exercise on cardiac and skeletal muscle myofibrillar proteins. *Mol. Cell. Biochem.* 83: 27-36, 1988.
5. Bellemare, F., and A. Grassino. Force reserve of the diaphragm in patients with chronic obstructive pulmonary disease. *J. Appl. Physiol.* 55: 8-15, 1983.
6. Cain, S. M. Oxygen delivery and uptake in dogs during anemic and hypoxic hypoxia. *J. Appl. Physiol.* 42: 228-234, 1977.
7. Collinson, P. O. Early diagnosis of myocardial infarction: why measure cardiac enzymes? *J. Clin. Pathol.* 51: 2-4, 1998.
8. Di Lisa, F., R. De Tullio, F. Salamino, R. Barbato, E. Melloni, N. Siliprandi, S. Schiaffino, and S. Pontremoli. Specific degradation of troponin T and I by μ -calpain and its modulation by substrate phosphorylation. *Biochem. J.* 308: 57-61, 1995.
9. Farah, C. S., C. A. Miyamoto, C. H. Ramos, A. C. da Silva, R. B. Quaggio, K. Fujimori, L. B. Smillie, and F. C. Reinach. Structural and regulatory functions of the NH_2 and COOH-terminal regions of skeletal muscle troponin I. *J. Biol. Chem.* 269: 5230-5240, 1994.
10. Ferrer, X., M. Coquet, J. Salntarailles, E. Ellie, B. Delplanque, C. Desnuelle, T. Levade, A. Lagueny, and J. Julien. Myopathie de l'adulte par déficit en maltase acide. Essai de traitement par régime hyperprotidique. *Rev. Med. Interne* 13: 149-152, 1992.
11. Fitting, J. W., F. Heritier, and C. Uldry. Évaluation de la force musculaire inspiratoire par la pression nasale lors du sniff. *Rev. Mal. Respir.* 13: 479-484, 1996.
12. Gao, W. D., D. Atar, Y. Liu, N. G. Perez, A. M. Murphy, and E. Marban. Role of troponin I proteolysis in the pathogenesis of stunned myocardium. *Circ. Res.* 80: 393-399, 1997.
13. Gelband, C. H., and H. Gelband. Ca^{2+} release from intracellular stores is an initial step in hypoxic pulmonary vasoconstriction of rat pulmonary artery resistance vessels. *Circulation* 96: 3647-3654, 1997.
14. Gorza, L., R. Menabo, F. Di Lisa, and M. Vitadello. Troponin T cross-linking in human apoptotic cardiomyocytes. *Am. J. Pathol.* 150: 2087-2097, 1997.
15. Jiang, T. X., W. D. Reid, A. Belcastro, and J. D. Road. Load dependence of secondary diaphragm inflammation and injury after acute inspiratory loading. *Am. J. Respir. Crit. Care Med.* 157: 230-236, 1998.
16. Laroche, C. M., J. Moxham, and M. Green. Respiratory muscle weakness and fatigue. *Q. J. Med.* 71: 373-397, 1989.
17. Levine, S., L. Kaiser, J. Leferovich, and B. Tikunov. Cellular adaptations in the diaphragm in chronic obstructive pulmonary disease. *N. Engl. J. Med.* 337: 1799-1806, 1997.
18. Lieber, R. L., L. E. Thornell, and J. Fridén. Muscle cytoskeletal disruption occurs within the first 15 min of cyclic eccentric contraction. *J. Appl. Physiol.* 80: 278-284, 1996.
19. Lowe, D. A., G. L. Warren, C. P. Ingalls, D. B. Boorstein, and R. B. Armstrong. Muscle function and protein metabolism after initiation of eccentric contraction-induced injury. *J. Appl. Physiol.* 79: 1260-1270, 1995.
20. McDonough, J. L., D. K. Arrell, and J. E. Van Eyk. Troponin I degradation and covalent complex formation accompanies myocardial ischemia/reperfusion injury. *Circ. Res.* 84: 9-20, 1999.
21. Ralenkotter, L., C. Dales, T. J. Delcamp, and R. W. Hadley. Cytosolic $[\text{Ca}^{2+}]$, $[\text{Na}^+]$, and pH in guinea pig ventricular myocytes exposed to anoxia and reoxygenation. *Am. J. Physiol. Heart Circ. Physiol.* 272: H2679-H2685, 1997.
22. Rama, D., I. Margaritis, A. Orsetti, P. Marconnet, P. Gros, C. Larue, S. Trinquier, B. Pau, and C. Calzolari. Troponin I immunoenzymometric assays for detection of muscle damage applied to monitoring a triathlon. *Clin. Chem.* 42: 2033-2035, 1996.
23. Reid, W. D., J. Huang, S. Bryson, D. C. Walker, and A. N. Belcastro. Diaphragm injury and myofibrillar structure induced by resistive loading. *J. Appl. Physiol.* 76: 176-184, 1994.
24. Roussos, C., and S. Zakynthinos. Fatigue of the respiratory muscles. *Intensive Care Med.* 22: 134-155, 1996.
25. Russ, U., H. Englert, B. A. Scholkens, and H. Gogelein. Simultaneous recording of ATP-sensitive K^+ current and intracellular Ca^{2+} in anoxic rat ventricular myocytes. Effects of glibenclamide. *Pflügers Arch.* 432: 75-80, 1996.
26. Sommer, L. Z., S. Iscoe, J. Silverman, J. Dickstein, A. Fink, A. Robicsek, D. Sommer, J. Kruger, A. Greenberg, G. Volgyesi, and J. A. Fisher. A simple breathing circuit minimizing changes in alveolar ventilation during hyperpnea. *Eur. Respir. J.* 12: 698-701, 1998.
27. Sorichter, S., J. Maier, A. Koller, W. Gebert, D. Rama, C. Calzolari, E. Artner-Dworzak, and B. Puschendorf. Skeletal troponin I as a marker of exercise-induced muscle damage. *J. Appl. Physiol.* 83: 1076-1082, 1997.
28. Takahashi, M., L. Lee, Q. Shi, Y. Gawad, and G. Jackowski. Use of enzyme immunoassay for measurement of skeletal troponin-I utilizing isoform-specific monoclonal antibodies. *Clin. Biochem.* 29: 301-308, 1996.
29. Thandroyen, F. T., D. Bellotto, A. Katayama, H. K. Hagler, J. T. Willerson, and L. M. Buja. Subcellular electrolyte alterations during progressive hypoxia and following reoxygenation in isolated neonatal rat ventricular myocytes. *Circ. Res.* 71: 106-119, 1992.
30. Tikunov, B. A., D. Mancini, and S. Levine. Changes in myofibrillar protein composition of human diaphragm elicited by congestive heart failure. *J. Mol. Cell. Cardiol.* 28: 2537-2541, 1996.



31. Van Eyk, J. E., F. Powers, W. Law, C. Larue, R. S. Hodges, and R. J. Solaro. Breakdown and release of myofilament proteins during ischemia and ischemia/reperfusion in rat hearts: identification of degradation products and effects on the pCa-force relation. *Circ. Res.* 82: 261-271, 1998.
32. Vassilakopoulos, T., S. Zakynthinos, and C. Roussos. Respiratory muscles and weaning failure. *Eur. Respir. J.* 9: 2383-2400, 1996.
33. Vincent, S. G., P. R. Cunningham, N. L. Stephens, A. J. Halayko, and J. T. Fisher. Quantitative densitometry of proteins stained with coomassie blue using a Hewlett Packard scanjet scanner and Scanplot software. *Electrophoresis* 18: 67-71, 1997.
34. Ward, M. E., H. Chang, F. Erice, and S. N. A. Hussain. Systemic and diaphragmatic oxygen delivery-consumption relationships during hemorrhage. *J. Appl. Physiol.* 77: 653-659, 1994.
35. Yanos, J., M. F. Keamy, III, L. Leisk, J. B. Hall, K. R. Walley, and L. D. H. Wood. The mechanism of respiratory arrest in inspiratory loading and hypoxemia. *Am. Rev. Respir. Dis.* 141: 933-937, 1990.

